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REPORT No.

J-288-2 (Final Report)

CONTRACT No.

DA-92-557-FEC-39983

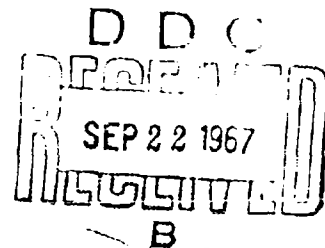
EXAMINATION FOR COMPLEMENT-REQUIRING NEUTRALIZING ANTIBODIES AGAINST  
JAPANESE ENCEPHALITIS, WESTERN EQUINE ENCEPHALITIS AND VACCINIA VIRUSES

by

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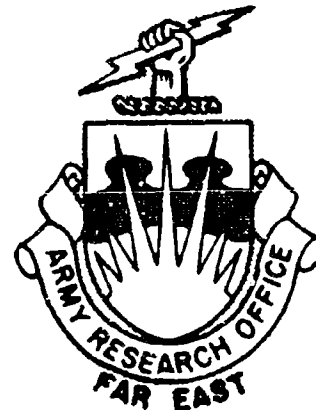
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September, 1967

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DA Project/Task Area/Work Unit No. 3A014301B71Q 00 0697E

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# ABSTRACT

It was planned to examine whether the early serum of animals immunized with Western equine encephalitis (WEE), Japanese encephalitis (JE) and vaccinia viruses contained complement-requiring neutralizing (CRN) antibody. A prerequisite to this experiment was to establish a simple neutralization test procedure which could titrate antibody titers of many serum samples at one time. Umeda et al's agar cover slip technique used for herpes virus neutralization was applied to WEE and JE viruses. When reaction mixtures were placed at 4°C overnight and then at 37°C for 3 hours before inoculation to cells, a clear endpoint could be obtained. With vaccinia virus, this method is not yet applicable. By the use of the above method, sera of guinea pigs immunized with WEE virus were subjected to neutralization tests in the presence and absence of 10 units complement, and it was shown that the CRN antibody appeared in the 2nd week serum. An exact estimation of the first detectable time of this antibody is being studied now. It seems that the appearance of the CRN antibody precedes that of HI and CP antibodies. In the cases of JE and vaccinia viruses, preliminary tests using rabbits clearly showed that the CRN antibodies appeared in early serums, and detailed tests are being done at present to determine the exact time of its appearance and the immunochemical nature of the CRN antibodies thereby obtained.

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## SECTION I

### Introduction

In the serodiagnosis of virus diseases, it has been a common practice to measure neutralizing, complement-fixing or hemagglutination-inhibiting antibody in the acute and convalescent serum samples to detect a titer rise between the paired serum samples. This procedure is especially necessary for endemic diseases, because normal healthy persons may possess a certain level of antibody when no special disease manifestation is taking place. Therefore, result of the serodiagnosis of virus diseases is obtained, in most cases, only after an advanced stage of infection or after death of the patient. Attempts to give an early diagnosis by other methods, e.g. virus isolation or fluorescent antibody staining of biopsied tissue or cells collected from urine, have not satisfied clinicians, since those procedures either require much time and special skill or are bothered by nonspecific reactions.

In the meantime, we found an interesting fact pertaining to the nature of the early antibody when studying the immune response of rabbits against herpes simplex virus. Namely, when rabbits were infected or immunized with herpes virus, there appeared early neutralizing antibody which could be detected only in the presence of a sufficient concentration of complement, whereas the late antibody did not require any complement for its detection (1,2,3). This was also the case with guinea pigs immunized with herpes virus (4). Then, sera of human patients suspected for herpes virus infections were examined, with the result that the complement-requiring neutralizing (CRN) antibody was detected invariably in the early serum of those who could be positively diagnosed by the conventional diagnostic procedures, while no such antibody was seen in sera of normal persons, whether or not persistently infected with herpes virus (4).

The above fact was of a great importance from the viewpoint of diagnosis, because it meant that only one serum sample taken at an early stage of infection might suffice to give positive diagnosis. It was also established that the CRN antibody against herpes virus was specific. The next problem to be pursued was whether CRN antibody appears in other virus diseases, too. Rawls et al. (5) recently reported that human

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antibody against rubella virus was enhanced by complement, but in this case both the early and late antibodies were enhanced equally. We intended to examine whether immunization with Western equine encephalitis (WEE), Japanese encephalitis (JE) or vaccinia virus stimulates the production of CMN antibody, and, if so, whether detection of such antibodies can be a tool for the early diagnosis of patients.

A preliminary experiment with these viruses indicated that in all these cases the CMN antibody appeared in the early serum of immunized animals.. For confirmation of those results with unequivocal results, a large scale experiment was planned for each virus. Thus far, we have determined (i) what technique of neutralization test is to be employed for each virus, and (ii) what animal and what immunizing process are suitable for detection of the CMN antibody of each virus.

## SECTION II

### Neutralization Test Procedure

In the planned experiments, it is expected to examine a large number of serum samples obtained from immunized animals in the presence and absence of complement. This kind of experiment requires a simplification of the neutralization test procedure, because the ordinary 50 % plaque reduction test needs much material and time and labor for performance. Earlier, Umeda et al. (6) worked out the agar cover slip (ACS) method for herpes simplex virus, in which virus-serum mixtures were inoculated to cells under cover slips made of overlay medium, and endpoints were determined by reading plaque patterns appearing at the inoculation spots, taking negative or few scattered plaques as positive neutralisation when the control spot showed confluent plaques. Advantages of this method were that one monolayer dish was enough to titrate each serum sample, and that reading of results was not time-consuming. The standard deviation of endpoints determined by this method was  $\pm 0.67 \log_2$  (2). Therefore, it was attempted to see whether this simple method could be applied to the present experiments with WEE, JE and vaccinia viruses.

1. WEE virus. When the above-stated ACS neutralization test was applied to WEE virus, however, first tests failed to demonstrate clear endpoints, because virus-serum mixtures contained comparatively high levels of unneutralized virus, especially in the neighborhood of the endpoint dilution of antiserum, and consequently inoculation of the mixtures under ACS did not result in a clear pattern discriminable from control. It was attempted, therefore, to lower the levels of unneutralized virus, in order to make feasible the application of this simple test to WEE virus.

a. Materials and methods. The following materials and methods were employed.

(1) Virus. McMillan strain of WEE virus was serially passaged through mouse brains and through chick embryo fibroblasts (CEF). The CEF line was cloned once to separate the small plaque variant (7). Passage histories and methods were detailed earlier (8). Virus dilutions were made with physiological saline containing antibody-free calf serum at 10 %.

(2) Antisera. Guinea pig antisera were produced by repeated intraperitoneal inoculations of infected CEF culture fluids inactivated with 1 : 3,000 betapropiolactone, and heated at 56°C for 30 minutes before storing at -20°C. Dilution of serum was done using 0.001 M  $MgCl_2$ -saline buffered with 0.0025 M Tris buffer at pH 7.8 (9).

(3) Plaque counting. The methods for preparation of CEF monolayers and plaque counting of WEE virus were the same as those for rabies virus stated elsewhere (8), except that the interval between the first and second (neutral red) overlayings was 2 days.

(4) Neutralization. Serial dilutions of antiserum were mixed with a constant amount of virus obtained from infected mouse brains; the virus suspension had been prepared with buffered saline containing 0.1 % bovine serum albumin and kept at -70°C in glass ampoules. After incubation of the mixtures as indicated, surviving virus amounts were titrated, using 3 monolayer dishes per dilution.

(5) ACS test. In this case, 90-mm dishes were used instead of the ordinary 60-mm dishes, and 10 ml of a cell inoculum had been sown to prepare monolayers. The overlay medium was solidified in metal boxes to make a thickness of 1.2 mm, and a puncher was applied to punch out ACS discs of 14 mm in diameter. An ACS was scooped on a flat metal spatulus

and 0.002 ml of a virus-serum mixture was placed on it by a platinum loop. The spatulus was turned upside down over a CEF monolayer and the edge of the ACS was pushed with forceps along the surface of the spatulus so that it dropped onto the cells gently without sliding on the cell surface. Eight ACS's were used per dish, and 8 ml of molten overlay medium was added for fixing of the ACS's. Subsequent procedures were the same as in the ordinary plaque counting.

b. Results. In the ACS technique, only one loopful of each virus-serum mixture is inoculated to cells and therefore a comparatively high concentration of virus should be used in reaction tubes. Whether this contributed to the high levels of unneutralizable virus in virus-serum mixtures was examined. The anti-WEE serum was diluted 1 : 20 to 1 : 2,560 in two series, setting plain diluent control for each series. One series received equal amounts of WEE virus so diluted as to make the final concentration of virus in the mixtures about  $1 \times 10^6$  PFU (plaque-forming units) per ml, while the other series was given a 500-fold dilution thereof. After incubation at 37°C for one hour in a water-bath, each mixture was titrated for survivors.

TABLE 1

Neutralization of 2 different concentrations of WEE virus by various concentrations of antiserum

Initial virus PFU/ml	Survivors in control PFU/ml	Log decrease of virus titer at dilution						
		80x	160x	320x	640x	1280x	2560x	
$1 \times 10^6$	$6.4 \times 10^5$	1.76	1.36	0.99	0.57	0.28	0.19	
$2 \times 10^3$	$1.2 \times 10^3$	1.62	1.62	1.22	0.66	0.22	0.18	

As can be seen in Table 1, the two virus concentrations left unneutralizable virus at similar ratios with all serum dilutions tested. The result from the lower virus series indicated that the endpoint as determined on the conventional criterion of 50 % plaque reduction was 1 : 640. When the serum diluent was buffered differently with Tris buffers from pH 7.2 to 9.0, no difference was observed in the above dose response curve, as shown in Table 2.

TABLE 2

Effect of different pH upon the dose response of antiserum against WEE virus

pH	Survivors in control PFU/ml	Log decrease of virus titer at dilution					
		80x	160x	320x	640x	1280x	2560x
7.2	$1.3 \times 10^6$	1.50	1.32	0.88	0.45	0.13	0.07
7.8	$1.2 \times 10^6$	1.70	1.19	0.85	0.29	0.13	0.08
8.4	$1.1 \times 10^6$	1.48	1.29	0.80	0.32	0.05	0.10
9.0	$1.1 \times 10^6$	1.58	1.38	0.76	0.31	0.17	0.03

In the next experiment, virus-serum mixtures were made in quadruplicate using an initial virus concentration of about  $1 \times 10^6$  PFU/ml, and titration of surviving virus was done after the following incubations: (i) one hour at 37°C, (ii) 3 hours at 37°C, (iii) overnight at 4°C and then one hour at 37°C, and (iv) overnight at 4°C and then 3 hours at 37°C. The result indicated in Table 3 shows that the last condition resulted in a sharp dose response curve in the neighborhood of the serum endpoint.

TABLE 3

Effect of different conditions of incubation of reaction mixtures upon the dose response curve

Incubation condition		Survivors in control PFU/ml	Log decrease of virus titer at dil.					
4°C	37°C		80x	160x	320x	640x	1280x	2560x
hr	hr							
0	1	$1.5 \times 10^6$	1.34	1.24	0.73	0.22	0.17	0.13
0	3	$9.8 \times 10^5$	1.58	1.49	1.33	0.81	0.26	0.08
24	1	$1.5 \times 10^6$	1.63	1.48	1.40	0.80	0.48	0.03
24	3	$1.2 \times 10^6$	1.57	1.63	1.69	1.20	0.69	0.25

Hence this condition of reaction was adopted in the final ACS test. Other factors influencing the result of this test were the virus dose and the plaque size. Discrimination of patterns between control (confluent plaques) and neutralization positive spots (negative or few scattered plaques) was easy when plaques were sufficiently small in size. The optimal conditions were determined as follows; the initial virus concentration (after mixing with serum) should be  $2.5 \times 10^7$  PFU/ml, and the incubation of inoculated dishes should be done at  $35^\circ\text{C}$  for 40 hours before adding the neutral red overlay. An example of the test performed under such conditions is shown in Fig. 1. It can be seen that the endpoint thus determined was the same as that obtained above by the 50 % plaque reduction method.



Figure 1. An example of the ACS neutralisation test with WEE virus.

2. JE virus. Experiences with JE virus were the same as with WEE virus. In this case, the overlay medium used contained 0.1 % bovine serum albumin in place of calf serum, and the interval between the two overlays was 4 days. It was determined that the initial virus concentration in reaction mixtures should be  $1 \times 10^6$  PFU/ml, and the interval between the first and second overlayings of ACS-inoculated dishes should be 3 days. Fig. 2 illustrates an example of such tests.

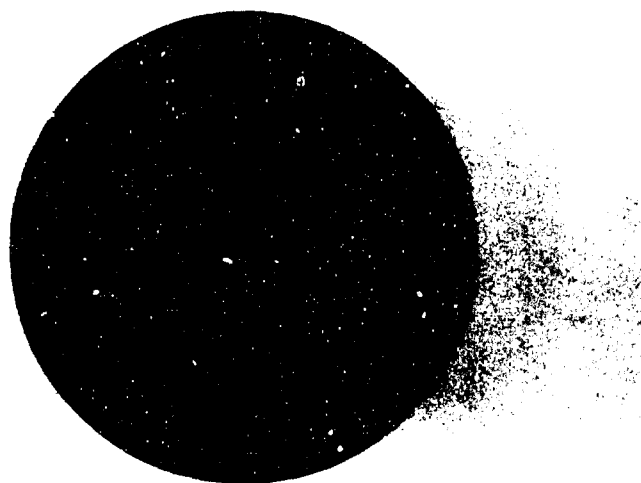


Figure 2. An example of the ACS neutralization test with JE virus.

3. Vaccinia virus. The above ACS test could not be applied to vaccinia virus, because the unneutralizable fraction of this virus was too high. However, a recent report by Wallis and Melnick (10) has indicated that removal of virus aggregates by filtration could lower the unneutralizable fraction. We are now applying this result to the ACS test with vaccinia virus. Our result has not yet come out.

### SECTION III

#### The CPN Antibody of WEE Virus

1. Schedule of experiments. The materials and methods were essentially the same as stated under Section II-para. 1a, unless otherwise specified here.

a. Immunization and bleeding. A number of guinea pigs were examined for nonspecific virus inhibitors, and those which showed such inhibitors were excluded from the experiment. The rest were immunized with betapropiolactone-inactivated WFE virus, which was prepared as stated under Section II-para. 1a2, with weekly intervals, each time giving 1.0 ml of virus intraperitoneally per animal. Just prior to each immunization, 3 animals were selected randomly for bleeding, and their sera were pooled, inactivated and stored at  $-20^{\circ}\text{C}$ .

b. Absorption with normal CEF cells. Primary CEF cultures in large Roux bottles were monodispersed by treating with 0.02 % EDTA plus 0.05 % trypsin, thoroughly washed with saline by centrifugation and resuspended in the above test sera at a concentration of  $10^6$  cells per ml. After one hour's standing at  $37^{\circ}\text{C}$ , each tube was transferred to an ice box. Next day, the cells were separated by centrifugation. This absorption was repeated three times to remove anti-cellular antibody.

c. Complement. The source of complement was normal guinea pig serum. Since some guinea pigs possessed heat-labile nonspecific virus inhibitors, such sera were omitted after preliminary tests. Complement, as well as antiserum, was diluted with pH 7.8 Tris buffered saline (Section II-para. 1a2). Determination of complement units was detailed earlier (2).

d. ACS neutralization test. This has been described in detail under the preceding Section. In the present experiments, 0.2 ml of serially diluted serum was kept in an ice bath, then given 0.1 ml of complement representing 10 hemolytic units, and finally added with 0.1 ml of virus representing  $1 \times 10^6$  PFU/ml, so that the final concentration of virus therein was  $2.5 \times 10^6$  PFU/ml. Control series received diluent only in place of complement. Other details of the procedure are to be referred to the preceding Section.

2. Results. The first experiment yielded the result tabulated in Table 4. For each serum, the test was done in duplicate, and the titers obtained are all recorded in the table. It may be obvious that the 2-week serum was enhanced by complement to a marked extent, in a sharp contrast to late serum samples which could not be enhanced by complement so much.

TABLE 4

Duplicate ACS neutralization tests of anti-WEE guinea pig sera in the presence and absence of complement

Time after first im- munization	Titer*		Titer*	
	without C'		with C'	
weeks				
0	0,	0,	0,	0,
2	40,	160,	2,560,	5,120,
4	160,	1,280,	1,280,	1,280,
6	2,560,	2,560,	10,240,	10,240,
8	1,280,	1,280,	5,120,	5,120,
12	1,280,	1,280,	2,560,	5,120,

(\*: Reciprocal of endpoint dilution)

## SECTION IV

## The CRN Antibody of JE Virus

The experiments with JE virus met some difficulties which did not appear in the case of WEE virus. In the first place, guinea pigs as well as rabbits purchased here were mostly very likely to have been bitten by mosquitoes during the preceding year, and therefore preliminary screenings for antibody often showed the presence of pre-existing antibody. Even when there was no pre-existing antibody, we were not certain that those animals had not been infected by JE virus previously by mosquito bite, and as a consequence it was difficult to determine whether the immune response studied was of the primary or secondary nature. In the second place, guinea pig serum to be used as complement very frequently contained specific antibody. Hence, it was necessary to bleed young guinea pigs born during winter to obtain usable complement.

To overcome these difficulties, we postponed the immunization of animals until this early summer, by which time a careful preliminary test for pre-existing antibody was done with a number of rabbits and guinea pigs. The immunization process was so scheduled that it could be finished before mosquitoes harboring JE virus appear in this district. The immunogen used was live and betapropiolactone-inactivated JE virus obtained from infected mouse brains. The result has not yet come out.

## SECTION V

### The ORN Antibody of Vaccinia Virus

Since, as stated above, the simple ACS test could not be used in the case of vaccinia virus, the 50 % plaque reduction neutralization test was performed with sera of rabbits, which were immunized with live vaccinia virus (Deiren I strain, HeLa cell-adapted) first by intradermal injections and later by an intravenous booster injection.

1. Schedule of experiment. Rabbits which showed no pre-existing antibody were given HeLa grown vaccinia virus intradermally at 8 spots, each with 0.1 ml. of virus suspension containing  $3.2 \times 10^7$  PFU/ml. (PFU was determined in CEF cells.) The plaque counting of this virus was done in the same manner as that for herpes virus previously established (11). Ten weeks later, a second injection of the virus was done intravenously giving 0.1 ml of a virus suspension containing  $3.2 \times 10^7$  PFU/ml. Sera obtained at 1, 3, 6 and 12 weeks following the first immunization were examined for neutralizing antibody. Reaction mixtures were prepared as stated in Section III-para.1d, and after one hour's incubation at  $37^\circ\text{C}$  inoculated to 3 CEF monolayers per mixture.

2. Result. Result of this experiment is recorded in Table 5. It can be seen that up to the 6th week a certain grade of enhancement of antibody titer by complement was seen. Detailed examination of these sera is now under way.

TABLE 5

Plaque reduction neutralization test with anti-vaccinia rabbit sera in the presence and absence of complement

Time after the first immunization	C <sup>o</sup>	Average plaque number at serum dilution					Cr
		5x	40x	320x	2,560x	20,480x	
weeks							
0	-	72					57
	+	26					31
1	-	60	66	68	88		
	+	27	28	37	39	25	
3	-	21	29	56	77		
	+			13	24	35	
6	-	31	58	71	61		
	+			13	28	34	
12	-	5	19	32	60		
	+			7	12	26	

## SECTION VI

## Discussion

Ever since we established that the detection of CRN antibody against herpes simplex virus could serve for the early diagnosis of diseases caused by this virus, several other workers have tested whether this is also the case with other viral infections. Rawls and Melnick (5) indicated that in the case of rubella virus infections both the early and late sera are equally enhanced by complement, therefore the detection of CRN antibody did not serve for an early diagnosis. Nishimura et al. (12) immunized rabbits with vaccinia virus and found that specific CRN antibody appeared during an early stage of immunization. He emphasized that this new method could be a good tool for an early serodiagnosis of varicella patients.

With JE virus, results of several workers are somewhat confusing. First, Prince and Hashimoto (13) described that no enhancement of neutralizing antibody titer by complement was seen in the case of sera of rabbits immunized with JE virus. Later, Iwazaki (14) demonstrated that IgM in the early serum of rabbits immunized with JE virus contained complement-potentiatable antibody, which was largely nonspecific and reactable with cellular (PS-Y cells) antigen. Finally, Ozaki (15) indicated that both the early and late sera of immunized rabbits were strongly enhanced by complement. These discrepancies seem to have stemmed from the different antigens used for immunization and different cells used in the neutralization test procedure.

It is necessary, in general, to use different sources of virus for immunizing antigen and for in vitro test, because otherwise an antigen-antibody system other than the virus-antivirus may cause reaction in the in vitro test and fix complement. Therefore, in such a case, a false negative result will be given when complement-potentiable reaction exists. Secondly, if the antiserum contains anti-cellular antibody (16, 17, 18) and the plaque reduction test is done in the cells which are subject to its influence, another false result might confuse the result. To avoid these confusions, we adopted systems in which no consideration on those factors are necessary. Namely, for WEE virus, guinea pigs were immunized with CEF-grown virus and the sera obtained were absorbed with normal CEF cells thoroughly; and for the in vitro neutralization test, virus from mouse brains was used. In the case of JE virus, rabbits were immunized with virus from infected mouse brains, and for the in vitro test, the CEF-grown virus is used; the plaque reduction was tested with CEF cells. For vaccinia virus, rabbits were infected and immunized with HeLa-grown virus and the in vitro test was done with the chorioallantoic membrane-grown virus using the CEF monolayers.

With the above care taken, the preliminary tests showed the presence of the CRN antibody for all the three viruses tested. The exact time of appearance of the CRN antibody, its relation to the immunizing dose of virus, the immunochemical nature of the CRN antibody, and its specificity are to be studied in more detail from now. In this study, it is planned to examine a large number of sera for neutralizing antibodies in the presence and absence of complement. Hence, a prerequisite to such an experiment was to establish a simple neutralization test procedure. This was accomplished in the

case of WEE and JE viruses by the use of the ACS neutralization test, and some preliminary results with WEE virus indicated the appearance of CRN antibody in an early stage of the immunization. A suitable method of neutralization test for vaccinia virus is now under study. We now think that application of the technique of Wallis et al. (10) which removes unneutralizable virus aggregates by filtration will enable the use of the ACS test in the case of vaccinia virus, too.

Since we have advanced mostly in the study of WEE virus, a discussion can be given as to the serological response against WEE virus. The guinea pigs immunized with WEE virus showed no HI antibody at the end of one week, but revealed a sharp increase of HI antibody after 2 weeks (19). The same sera, however, pointed out a high level of CRN antibody already at the end of the 2nd week. This means that the CRN antibody appeared earlier than HI antibody. In contrast, CF antibody was very low even in late stages of immunization. Thus, it may be said that the CRN antibody is the earliest of all antibodies. Hence, the detection of this antibody will certainly serve for early serodiagnosis. How early the first detectable time is must be determined in the subsequent study. Also, whether the same can be said in the cases of JE and vaccinia viruses must be determined.

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19. Yoshino, K. Progress Report J-288-1 of Contract DA-92-557-FEC-39983, January 1967.

Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Department of Bacteriology Yokohama City University, School of Medicine Yokohama, Japan		2a. REPORT SECURITY CLASSIFICATION Unclassified	
		2b. GROUP	
3. REPORT TITLE EXAMINATION FOR COMPLEMENT-REQUIRING NEUTRALIZING ANTIBODIES AGAINST JAPANESE ENCEPHALITIS, WESTERN EQUINE ENCEPHALITIS AND VACCINIA VIRUSES (U)			
4. DESCRIPTIVE NOTES (Type of report and including dates) Final Report, 25 June 1966 - 24 June 1967			
5. AUTHOR(S) (First name, middle initial, last name) Yoshino, Kamesaburo			
6. REPORT DATE September 1967	7a. TOTAL NO. OF PAGES 17	7b. NO. OF REFS 19	
8a. CONTRACT ORIGINATOR'S NO. DA-92-557-FEC-39983	8b. ORIGINATOR'S REPORT NUMBER(S) J-288-2		
8c. PROJECT NO. 3A014501B71Q			
8d. Task 00 069FE	8e. OTHER REPORT NUMBER (Any other numbers that may be assigned this report)		
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11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY U.S. Army R&D Group (Far East) APO San Francisco 96343	
13. ABSTRACT It was planned to examine whether the early serum of animals immunized with Western equine encephalitis (WEE), Japanese encephalitis (JE) and vaccinia viruses contained complement-requiring neutralizing (CRN) antibody. A prerequisite to this experiment was to establish a simple neutralization test procedure which could titrate antibody titers of many serum samples at one time. Umeda et al's agar cover slip technique used for herpes virus neutralization was applied to WEE and JE viruses. When reaction mixtures were placed at 4°C overnight and then at 37°C for 3 hours before inoculation to cells, a clear endpoint could be obtained. With vaccinia virus, this method is not yet applicable. By the use of the above method, sera of guinea pigs immunized with WEE virus were subjected to neutralization tests in the presence and absence of 10 units complement, and it was shown that the CRN antibody appeared in the 2nd week serum. An exact estimation of the first detectable time of this antibody is being studied now. It seems that the appearance of the CRN antibody precedes that of HI and CF antibodies. In the cases of JE and vaccinia viruses, preliminary tests using rabbits clearly showed that the CRN antibodies appeared in early serums, and detailed tests are being done at present to determine the exact time of its appearance and the immunochemical nature of the CRN antibodies thereby obtained. (Author)			

DD FORM 1473

1 JAN 64, March 64  
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	ROLE	WT	ROLE	WT	ROLE	WT
Antibodies Virus Diseases Tests results Cells						

Unclassified

Security Classification